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(54) Title: TAGGING OF RNA AMPLICONS GENERATED BY TRANSCRIPTION–BASED AMPLIFICATION

(57) Abstract

The present invention concerns a method for tagging the reaction products of transcription–based amplification procedures, and a kit for detecting such tagged nucleic acid amplified in a transcription–based amplification assay. The method is characterised in that RNA amplicons are generated by transcription–based amplification by introducing transcribable non–target related sequence elements in one or both primers used for transcription–based amplification which are transcribed into the RNA amplicons. In particular, the extra sequence elements thus introduced into the amplicons, can be used for the detection of the amplicons, for specific selection of the amplicons, to artificially extend the length of amplicons or to tag polypeptides translated from RNA amplicons generated by transcription–based amplification.

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TAGGING OF RNA AMPLICONS GENERATED BY TRANSCRIPTION-BASED AMPLIFICATION

The present invention concerns a method for tagging the reaction products of transcription-based amplification procedures, and a kit for detecting such tagged nucleic acid amplified in a transcription-based amplification assay.

Nucleic acid amplification methods are used in the field of molecular biology and recombinant DNA technology. These methods are used to increase the number of copies of a particular nucleic acid sequence, present in small amounts and often in an environment in which a wide variety of other nucleic acid sequences, both RNA and DNA, are also present. In particular, nucleic acid amplification methods are used to facilitate the detection or quantification of nucleic acid and are important for diagnosing for example infectious diseases, inherited diseases and various types of cancer. Nucleic acid amplification methods have also found their applications in other fields such as forensic sciences or archeology where samples are investigated in which nucleic acid may be present in minute amounts, or to establish paternity.

Several nucleic acid amplification techniques are known based on different mechanisms of action. One method for the amplification of nucleic acid known as the "Polymerase Chain Reaction" (PCR) is described in European patent applications EP 200362 and EP 201148. PCR is a cyclic process which has double stranded DNA as target. Each cycle in the PCR process starts with the separation of a double stranded DNA target in its two complementary strands. To each strand a primer will anneal and DNA polymerases present will extend the primers along the DNA strand to which it annealed thus forming two new DNA duplexes. When the reaction mixture is heated, the strands of the DNA duplexes will be separated again and a new PCR cycle can start. Thus, the PCR process produces multiple DNA copies of a DNA target. If single stranded RNA is the desired target for PCR, it has to be converted to double stranded DNA first by reverse transcriptase.

A different class of nucleic acid amplification methods are the "transcription-based amplification" techniques. The techniques involve the transcription of multiple RNA copies from a template comprising a promoter recognized by an RNA polymerase. With these methods, multiple RNA copies are transcribed from a DNA template that comprises a functional promoter recognized by the RNA polymerase. Said copies are used as a target again from which a new amount of the DNA template is obtained etc. Such methods have been described by Gingeras et al. in WO88/10315 and Burg et al. in WO89/1050. Isothermal transcription-based amplification techniques have been described by Davey et al. in EP 323822 (relating to the NASBA method), by Gingeras et al. in EP 373960 and by Kacian et al. in EP 408295. Transcription-based amplifications are usually carried out at a temperature around 41 degrees Celsius. Transcription-based amplification reactions may, however, also be performed with thermostable enzymes. These thermostable enzymes allow the reaction to be carried out at more elevated temperatures. Such a thermostable method is described in EP 682121 filed in the name of Toyo Boseki KK.

The methods as described in EP 323822, EP 373960 and EP 408295 are isothermal continuous methods. With these methods, four enzyme activities are required to achieve amplification: an RNA-dependent DNA polymerase activity, a DNA-dependent DNA polymerase activity, an RNase (H) activity and an RNA polymerase activity. Some of these activities can be combined in one enzyme so usually only 2 or 3 enzymes are necessary. Enzymes having RNA-dependent DNA polymerase activities are enzymes that synthesize DNA from a RNA template. A DNA-dependent DNA polymerase thus synthesizes DNA from a DNA template. In transcription-based amplification reactions, a reverse transcriptase such as AMV (Avian Myeloblastosis Virus) or MMLV (Moloney Murine Leukemia Virus) reverse transcriptase may be used. Such enzymes have both RNA- and DNA-dependent DNA polymerase activity but also an inherent RNase activity. In addition an RNase may be added to the reaction mixture of a transcription-based amplification reaction, such as E. coli RNase H.

DNA-dependent RNA polymerases synthesize multiple RNA copies from a DNA template including a promoter recognized by the RNA polymerase. Examples of RNA polymerases are polymerases from E. coli and bacteriophages T7, T3 and SP6. An example of an RNA polymerase commonly used with transcription-based amplification

methods is T7 RNA polymerase. Thus, the promoter that is incorporated in the template used for transcribing multiple copies of RNA would then be the T7 promoter. Usually, the template comprising the promoter has to be created starting from the nucleic acid comprising the target sequence. Said nucleic acid may be present in the starting material that is used as input for the amplification reaction. The nucleic acid present in the starting material will usually contain the target sequence as a part of a much longer sequence. Additional nucleic acid sequences may be present on both the 3'- and the 5'-end of the target sequence. The amplification reaction can be started by bringing together this nucleic acid from the starting material, the appropriate enzymes that together provide the above mentioned activities and at least one, but usually two, oligonucleotide(s). At least one of these oligonucleotides should comprise the sequence of the promoter.

Transcription-based amplification methods are particularly useful if the input material is single stranded RNA, although single or double stranded DNA can likewise be used as input material. When a transcription-based amplification method is practiced on a sample with single stranded RNA (of the "plus" sense) with additional sequences on both the 3' end and the 5' end of the target sequence a pair of oligonucleotides that is conveniently used with the methods as described in the prior art would consist of:

- a first oligonucleotide ("primer", usually also referred to as "promoter-oligonucleotide") that is capable of hybridizing to the 3'-end of the target sequence, which oligonucleotide has the sequence of a promoter (preferably the T7 promoter) attached to its 5' end (the hybridizing part of this oligonucleotide has the opposite polarity as the plus RNA used as input material).
- a second oligonucleotide ("primer") which comprises the 5' end of the target sequence (this oligonucleotide has the same polarity as the plus RNA).

When such a pair of oligonucleotides and the target nucleic acid, together with all enzymes having the appropriate activities, and a sufficient supply of the necessary ribonucleotides and deoxy-ribonucleotides are put together in one reaction mixture and are kept under the appropriate conditions (that is, under the appropriate buffer conditions and at the appropriate temperature) for a sufficient period of time, an isothermal continuous amplification reaction will start.

Techniques for the detection of amplified nucleic acid are well known in the art. Most commonly, such detection techniques comprise the introduction of a label directly into the amplificate. A detection may include an additional hybridisation step in order to increase the specificity of the assay. Analysis of transcription-based amplification reaction mixtures may require separation of the target nucleic acid-derived amplicons and the non-specific reaction products. One example by which this can be achieved is subjecting the sample with amplified nucleic acid to gel electrophoresis. Following separation by gel electrophoresis, the gel can be blotted onto a filter and hybridised with a complementary oligonucleotide labelled with a detection moiety. These kind of detection methods make use of the length of the RNA amplicons in combination with their specific primary structure. To circumvent cumbersome gel electrophoresis, other detection methods have been developed with the advantage of a much higher throughput. In these detection formats, target nucleic acid-derived amplicons are captured onto a solid phase by hybridisation to a complementary oligonucleotide immobilised on the solid phase. Subsequently, the captured amplicons can be detected by a moiety labelled with a reporter molecule.

One example of nucleic acid analysis making use of such sandwich hybridisation principle is electrochemiluminescence-based (ECL-based) detection as described by Kenten et al., Clin. Chem. 38, pp 873-879 (1992). The solid phase is formed by paramagnetic beads and target nucleic acid-derived amplicons bound by specific hybridisation to a complementary oligonucleotide immobilised on the beads can be detected by a second complementary oligonucleotide labelled with the ruthenium chelate Tris(2,2'-bipyridine)ruthenium(II) (TBR). Upon hybridisation, the beads carrying the amplicon/TBR-labelled oligonucleotide complex are brought to the surface of an electrode by a magnet. When a low voltage is applied, a cyclical oxidation-reduction reaction is triggered in which the ruthenium chelate in the presence of tripropylamine (TPA) produces light. In this process, the TPA is consumed whereas the ruthenium chelate is recycled.

Methods for the introduction of a label directly into the amplified nucleic acid, i.e. covalently bound to the reaction product, are well known in the art. In PCR, one of the primers can be labelled with a suitable detectable moiety which in turn is then

incorporated into the amplification product. Useful labels include radioisotopes, fluorescent moieties, enzymes or specific binding moieties such as avidin or biotin, as noted for example in US Patent no US-A-4,879,214. The use of biotinylated oligonucleotide primers in the PCR amplification reaction, for example, reveals biotin-labelled end products, which upon binding to a solid phase by hybrid-capture, can be detected by streptavidin complexed to a reporter molecule like horse radish peroxidase (HRP) or a fluorescent moiety.

In contrast to PCR, the oligonucleotide primers employed in a transcription-based amplification reaction do not form an intrinsic part of the single stranded antisense RNA which is generated as the major amplification product. Reaction products from transcription-based amplification reactions, therefore, cannot be labelled by performing the reaction with labelled primers. In order to obtain directly labelled end products in transcription-based amplification reactions, the use of labelled ribonucleotide-5'-triphosphates has been suggested (IBC UK conferences Ltd Functional Genomics: enabling technologies for drug discovery November 12 & 13, 1997, London Conference Documentation). A drawback of these complexed ribonucleotides is that they are poor substrates for RNA polymerases, thereby compromising the sensitivity of transcription-based amplification. For this reason, hybridisation assays are preferred for the detection of reaction products of transcription-based amplification reactions.

A specific hybridisation probe has to be newly developed for each target, based on the particular nucleic acid sequence of that target. This may be a cumbersome, time consuming and expensive procedure, especially if one wants to amplify a large number of different targets or if one wants to use a detection probe with a label such as an ECL label covalently attached. The present invention provides a solution to that problem in that it provides a method for the introduction of a (generic) sequence element into the reaction products of a transcription-based amplification reaction in order to be able to detect that specific element with a (generic) detection probe, independent of the nucleic acid sequence of the target. Such a specific element is herein referred to as a transcribablenon-target related sequence element.

The method of the present invention includes the feature that where for transcription-based amplification methods introduction of labelled primers or mononucleotides is not possible or difficult to achieve, the major amplification product (i.e. single stranded antisense RNA) can be tagged with a certain ribonucleotide segment which is introduced into the amplification product during transcription by making use of a primer which in addition to the target RNA-derived sequence contains an extra non-target related sequence element. A person skilled in the art would expect that introduction of a transcribable non-target related sequence element into one or both primers would negatively influence the amplification efficiency of such primers when used in a transcription-based amplification assay. Surprisingly, it was found that neither the length of such sequence element nor its primary structure appeared to have any impact on the performance of the primer in the amplification reaction.

The tag consisting of a stretch of nucleotides can either be added at the 5' end of the upstream primer or between the target RNA-derived sequence and the RNA polymerase promoter segment of the downstream primer, resulting in a tag at the 3' end or the 5' end of the RNA amplicons, respectively. Use of a combination of tagged upstream and downstream primers is possible as well, introducing an extra ribonucleotide segment at either end of the RNA amplicon.

The invention thus concerns a method for tagging the reaction products of a transcription-based amplification assay, comprising the steps of adding to a reaction mixture;

a. at least one oligonucleotide that can function as a nucleic acid primer for a transcription-based amplification reaction
b. appropriate enzymes
c. target sequence

and subsequently incubating the mixture at an appropriate temperature for an appropriate amount of time in order to accomplish amplification of at least part of the target sequence characterised in that said at least one oligonucleotide contains a transcribable non-target related sequence element.

In a preferred embodiment, said transcription-based amplification assay is a NASBA assay.

In a further preferred embodiment, said method is characterised in that two oligonucleotides contain a transcribable non-target related sequence element.

In an even more preferred embodiment, said method is characterised in that said transcribable non-target related sequence element comprises a nucleic acid sequence selected from the group consisting of SEQ ID # 1-4.

Upon amplification, presence of target nucleic acid-derived amplicons generated in the amplification reaction is usually demonstrated by detecting the amplicons with a labelled probe which can specifically hybridise to the amplicons. It was now found that amplicons could be detected with equal efficiency, sensitivity and specificity irrespective whether a target related nucleic acid sequence or a non-target related sequence, introduced by the above described method, was targeted by the detection probe. Therefore, extension of oligonucleotides used as primers in transcription-based amplification reactions and subsequent detection of the generated amplicons by means of hybridisation of an oligonucleotide complementary to the sequence element that is thus introduced into the amplicons, is feasable and enables the use of a generic detection moiety for target nucleic acids of different origin.

The present invention can thus be used for the detection of nucleic acids in a sandwich hybridisation assay in which either the capture probe or the detection probe is complementary to the extra RNA segment introduced into the RNA amplicon. If a universal tagging segment is used for different target RNAs, instead of two target RNA-derived oligonucleotides, one of which functions as the capture probe and the other as the detection probe, only one target RNA-derived oligonucleotide is needed in combination with a second generic oligonucleotide which is complementary to the tag of the RNA amplicons.

A preferred embodiment of the present invention is therefore a kit for detecting nucleic acid amplified in a transcription-based amplification assay using the above

described method, comprising a nucleic acid probe hybridisable to the transcribable non-target related sequence element or its complement.

In a further preferred embodiment said nucleic acid probe is attached to a detectable label.

In an even more preferred embodiment said detectable label is an ECL label.

The present invention can also be used to separate amplicons that carry the extra non-target related sequence from those that do not. Tailing of the upstream primer with a transcribable, non-target-related sequence element reveals RNA amplicons with the non-target related sequence element at their 3' ends. As synthesis of the RNA amplicons in transcription-based amplification reactions occurs in the 5' end to 3' end direction, as a consequence, only full-length run-off transcripts contain the non-target related sequence element. Those can than be separated from truncated transcription products through hybrid capture employing an oligonucleotide that is complementary to the non-target related sequence element. This might be especially useful when transcription-based amplification is followed by translation of the generated amplicons into a polypeptide. Selection of full-length transcripts prior to translation greatly enhances the production of full-length translation products as translation of abortive transcripts is abolished.

The method of the present invention can also be used for tagging a polypeptide with a certain stretch of amino acids such as, for example, hexa-histidine for metal-chelate separation or with an epitope sequence for separation by an immobilised monoclonal antibody (for a review see Jones et al., J. Chromatogr. A 707, pp 3-22 (1995)). This can be achieved by chosing the correct coding sequence for the extra sequence element added to one of the primers used for amplification. Using ordinary transcription of a DNA fragment for the production of RNA transcripts meant for subsequent translation into a polypeptide does not offer the possibility of introducing a tag in the resulting polypeptide other than by manipulating the DNA fragment from which the RNA is transcribed with molecular cloning techniques well known in the art. By generating RNA transcripts for subsequent translation through transcription-

based amplification, tagging of the resulting polypeptide can much easier be achieved, simply by elongation of one or both primers with an appropriate sequence element. Changing the tag of the polypeptide can be achieved by changing the primary structure of the extra sequence element added to the primer or primers used, thereby circumventing the need to generate a new DNA clone which would be much more labour intensive.

Thus, the method of the invention enables the generic labelling of amplicons. Due to the presence of the generic sequence in the amplicons, the method of the invention also enables the differentiation of contaminating amplicons and target molecules in subsequent amplification reactions.

The amplicons generated by the method of the invention will all comprise a generic sequence. To counteract contamination of amplicons during handling of samples in pre-amplification steps (e.g. nucleic acid isolation) the generic sequence, that will only be present in contaminating amplicons and not in the target sequence, can be used to separate the contaminating amplicons from the sample.

Thus, the present invention further comprises a method for counteracting carry-over contamination in transcription based amplification reaction mixtures. Said method comprises tagging the reaction products of a transcription based amplification reaction by adding at least one oligonucleotide that can function as a nucleic acid primer for a transcription-based amplification reaction to the amplification reaction mixture. Further reaction mixtures can then be checked, prior to amplification, for the presence of tagged reaction products., Said tagged reaction can optionally be removed from the reaction mixture or kept separate from the amplification reaction. The tagged reaction products may be removed by reacting them with a capture-oligonucleotide comprising a complementary sequence. Such capture oligonucleotides are preferably bound to a solid phase.

The solid phase may then be contacted with the reaction mixture under conditions suitable for binding the tagged reaction products to the capture-oligonucleotides. When the tagged reaction products are bound to the capture oligonucleotides, they can easily be removed from the reaction mixture or be kept aside during the amplification reaction so that the tagged products with which the sample was contaminated will not participate in the amplification reaction. To bind the tagged

reaction products with which the reaction mixture optionally is contaminated to the solid phase the reaction mixture, or the sample from which the reaction mixture is prepared may be contacted with the solid phase during work up. For example, during nucleic acid isolation from the sample, but in any event prior to amplification. The solid phase may stay in touch with the reaction mixture during primer addition, and can be separated from the pre-amplification mixture prior to addition of the enzymes necessary for the amplification reaction.

The solid phase may have any suitable format. For example, the solid phase may be particulate. The particles, with the tagged reaction products bound thereto, may than be separated from the reaction mixture by filtration.

The solid phase may also be composed of magnetic or (super)paramagnetic beads. In that case the solid phase can be separated from the reaction mixture using a magnet.

The present invention enables the use of generic components in an amplification kit. That is, with the method of the invention, reaction products of a transcription based amplification reaction can be tagged with a sequence that has nothing to do with the sequence of the specific target, i.e. a "generic" sequence", and thus, a detection kit based on said method may, for example, comprise a labeled probe, comprising an oligonucleotide with a sequence that is the complementary of the generic sequence introduced in the reaction products of a transcription based amplification method. Thus, the probe can be used in many assays, irrespective of the target sequence or organism to be detected.

The same principle can also be used to construct internal standards for transcription based amplification reactions. In qualitative as well as quantitative transcription based amplification reactions internal standards are often used. Either to be able to check whether the amplification reaction was carried out correctly or to have a known amount of standard material based on which the amount of a target sequence present in a sample can be determined. For example, the use of internal standards in transcription based amplification reactions is described in EP656955. Quantitative transcription based amplification methods wherein internal standards are co-

amplified with the target, using the same primers are described in EP525882 and EP662156.

In transcription based amplification based kits, these internal standards are provided to the user and are specific for the target sequence to be amplified, since the standards have to comprise the same primer binding sites as the target sequence within the nucleic acid of the organism to be detected with the kit.

In some instances however, it may be useful for the user of a transcription based method, to be able to prepare it's own internal standards For example, the user of a kit according to the invention, might not be provided with internal standards with said kit, simply, because, while the kit is generic, the standards would have to be target specific.

Thus, as a further embodiment of the present invention, a method is provided for the generation of RNA transcripts that can be used as internal standard in a transcription based amplification reaction. With this method an internal standard sequence is tagged with transcribable non-target related sequence elements. In this case, however, the transcribable non-target related sequence elements are used to introduce the target specific primer binding sites into generic internal standards, for the amplification reaction in which the internal standards are to be used.

The sequence of the internal standards between the primer binding sites can be chosen by the user. It may resemble the target sequence, but will include specific regions to be able to discriminate it from the target, for example, specific probe binding regions.

The internal standards may have the same length as the target or may differ in length from the target sequence.

Since, eventually, the internal standard will be co-amplified with the target, they can, in the amplification reaction, be tagged with a transcribable non-target related sequence element, for example, to allow them to hybridize with the same generically labelled probe as the target.

The primer binding sites for the eventual amplification reaction (for the sake of clarity referred to as "A" and "B") in which the internal standards are to be used can be introduced trough transcription based amplification reactions with primers that include the sequences "A" and/or "B" or the complement thereof as an additional sequence, i.e. as a transcribable non-taget related sequence element.
An optional reaction scheme for introducing "A" and "B" or the complement thereof into internal standards is illustrated in figure 2.

One way of creating nucleic acid molecules that can be used as internal standards including primer binding sites A and B is by performing two rounds of transcription based amplification
The RNA on which the internal standards are based will be referred to as "generic input RNA". As stated above, said RNA may have any sequence desired by the user. It may resemble the target is certain ways, but will be distinguishable therefrom as well. For example, because it has its own specific probe binding site, or because it comprises a mutation based on which it can be distinguished from the target (or rather; the amplification products derived therefrom) or because it differs in length from the target.
This generic input RNA may be subjected to a first transcription based amplification reaction wherein a first and second primer are used and said first primer comprises a sequence homologous to the 5' region of the generic input RNA and further comprising a 5' tail, said 5' tail comprising a primer region A homologous to the target RNA, and a second primer comprising a sequence complementary to the 3' end of the generic input RNA, said second primer further comprising a sequence of a promoter recognised by an RNA polymerase. With the use of these primers in a first round of a transcription based amplification reaction, products are created that have a primer A region at their 3' end.
Subsequently these reaction products, including the primer A region, may be subjected to a second transcription based amplification reaction wherein a third and fourth primer are used. In this second round the primer B region is introduced. Said third primer comprises the sequence of the primer A region and the sequence of a promoter for a RNA polymerase. Thus this primer will bind to the primer A region present in the reaction product of the first round of amplification.

Said fourth primer comprises a sequence complementary to the 3' region of the generic input RNA and further comprises a sequence homologous to the primer B region. In the second round of transcription based amplification using the third and fourth primers thus, as a reaction product, internal standards are produced that have a primer A binding region and a primer B region. The primer A region and primer B region are also present in the target RNA and thus these internal standards can now be used in an amplification reaction together with the target. Either as an internal control for the reaction or as a calibrator in a quantitative reaction. It will be evident that many variations on the above described reaction scheme are possible, all based on the same principle, namely the introduction of the primer binding sites by using them as a "transcribable, non-target related sequence element" in the method of the invention, and that all these variations are likewise part of the present invention.

In general, there is a lower limit with regard to the length of the target nucleic acid sequence in transcription-based amplification methods. In NASBA, for example, a minimal length of at around 100 nucleotides is preferred for efficient amplification of the target nucleic acid sequence. If such a minimal length is not available, the length of the amplification product can artificially be extended by introducing extra non-target related sequences into one or both primers used for transcription-based amplification of that specific target nucleic acid. A person skilled in the art would recognise that the above is especially useful in one or more of the following situations. For example, when a conserved sequence element of limited length which would be extremely suitable for amplification-based detection due to its high degree of sequence conservation is flanked by hypervariable regions not enabling extension of the segment to be amplified without compromising the sensitivity of the assay. Or if one would want to amplify relatively short RNAs like the recently described 3' non-coding terminal sequence of the hepatitis C virus (HCV) genomic RNA as described by Kolykhalov et al., J. Virol. 70, pp 3363-3371 (1996), which is highly conserved but only 98 nucleotides in length. In the HCV genome, this 3' terminal sequence is preceded by homopolymer tracts of either poly(U) or polypyrimidine stretches consisting of mainly U residues with occasional interspersed C residues. For reasons of specificity, homopolymeric sequences are not well suited as a primer annealing site. Therefore, the target segment for amplification cannot be expanded beyond this

3' terminal stretch of 98 nucleotides which by itself has not an ideal length for efficient transcription-based amplification. Therefore, artificial extension of the target segment by elongation of one or both primers can be beneficial for more efficient transcription-based amplification.

Brief description of the figures

Figure 1: The figure shows a photographic representation of an enzyme-labeled gel assay (ELGA) wherein primers with a transcribable non-target related sequence elements are analysed for their performance in a NASBA assay. P2: U1A P2 primer with 5' end elongations of different sizes. U1A P2 primers with elongations of 15 (P2 + 15), 20 (P2 + 20) and 24 (P2 + 24) nucleotides were were compared to a standard P2 primer (P2) for the amplification of U1A Sc-RNA. Reaction products were analysed by ELGA. Lanes: (10^4) 10^4 molecules of U1A SC-RNA in the amplification reaction; (10^3) 10^3 molecules of U1A SC-RNA in the amplification reaction; (10^2) 10^2 molecules of U1A SC-RNA in the amplification reaction; (10^1) 10 molecules of U1A SC-RNA in the amplification reaction.

Figure 2: Preparation of internal standards using two rounds of transcription based amplification.

The invention is further exemplified by the following examples:

Examples

Example 1: Primers and probes.

Oligonucleotide primers and probes were synthesised on a PCR-MATE 391 DNA synthesiser (Applied Biosystems) using phosphoramadite biochemistry.

Amplification primers were purified by electrophoretically separating the crude oligonucleotide solutions over a 20% polyacrylamide/7M Urea slabgel and subsequent elution of the full-length oligonucleotide from the corresponding gel band. After elution from the gel slices and concentration by ethanol precipitation,

primers were dissolved in Milli-Q water and concentrations determined by OD(260 nm) measurement.

Oligonucleotide probes for ELGA detection were synthesised with a 5'-amino link (Aminolink 2; Applied Biosystems) and conjugated with Horse Radish Peroxidase (Boehringer) by coupling the enzyme to an amino link oligonucleotide using the cross-linking reagent SPDP (Pharmacia). Unbound HRP was removed by passing the reaction mixture over a Qiagen Tip-100 column (Qiagen). The HRP-labelled oligonucleotide was further purified by slabgel electrophoresis. The amount of HRP-conjugated oligonucleotide was calculated from OD(260 nm) and OD(400 nm) measurement.

5' Biotin-derivatized oligonucleotides were synthesised using Biotin Amadite (Applied Biosystems).

For ECL detection, oligonucleotide probes were synthesised with a 5'-amino link and conjugated with the ECL label Tris(2,2'-bipyridine)ruthenium(II) (TBR) by incubating the amino link oligonucleotides with TAG NHS-Esther (Igen). Unbound label was removed by passing the reaction mixture over a Qiagen Tip-100 column (Qiagen). The amount of ECL-labelled oligonucleotide was calculated from OD(260 nm) and OD(460 nm) measurements. The solutions were stored at -70° C and used without further purification.

Example 2: Isolation of total nucleic acid from blood compartments.

For nucleic acid isolation from different blood compartments, one volume of plasma or EDTA-anticoagulated whole blood was added to 9 volumes of Lysis buffer [50 mM Tris-Hydrochloric acid (pH 6.4); 20 mM EDTA; 1.3 % (w/v) Triton X-100; 5.25 M Guanidine thiocyanate]. Typically, 100 µl of plasma or 1 ml of whole blood were used. To the lysed plasma or whole blood suspensions in vitro generated target RNA was spiked prior to the addition of 50 µl of hydrochloric acid-activated silicium dioxide particles (Sigma) [size-selected suspension of 1 mg/ml in 0.1 M Hydrochloric acid; see Boom et al, J. Clin Microbiol. 28, pp 495-503 (1990). The suspension was incubated during 10 minutes at room temperature with regular vortexing. Nucleic acid bound to the silica was spun down by centrifugation. Pelleted silica particles were washed twice with 1 ml GuSCN wash buffer [50 mM Tris-Hydrochloric acid (pH 6.4); 5.25 M Guanidine thiocyanate], followed by two washing steps with 1 ml 70 %

ethanol and a single washing step with 1 ml acetone. After each washing step, the suspension was briefly centrifuged and the silica pellet was resuspended in the next washing solution by thorough mixing. After removal of the acetone, the silica particles were dried by incubation at 56° C in a heating block during 10 minutes. Nucleic acid was eluted from the silica particles by incubation in 50 µl Elution buffer [1 mM Tris-Hydrochloric acid (pH 8.5)] at 56° C during 10 minutes. Finally, the silica particles were spun down again and the supernatant was carefully pipetted into fresh reaction tubes avoiding any carry-over of silica. Extracted nucleic acid samples were stored at -70° C until use.

Example 3: NASBA amplification.

To set up a NASBA amplification reaction, a premix was generated by reconstituting a lyophilised Primer accusphere containing the necessary ingredients for a NASBA reaction in Primer diluent. Of this premix [80 mM Tris-Hydrochloric acid (pH 8.5); 140 mM Potassium chloride; 24 mM Magnesium chloride; 10 mM DTT; 2 mM of each dNTP; 4 mM of ATP, CTP and UTP; 3 mM of GTP; 1 mM of ITP; 3% (w/v) Sucrose; 1% (w/v) Mannitol; 1% Dextrane T40; 0.4 µM of each primer], 10 µl was added to 5 µl nucleic acid solution and incubated during 5 minutes at 65° C. Subsequently, the reaction tubes were incubated at 41° C during 5 minutes before 5 µl Enzyme mix [32 units T7 RNA polymerase; 6.4 units AMV reverse transcriptase; 0.08 unit RNase H; 2.1 µg BSA; 20 mM DTT; 1.5 M Sorbitol] was added. After the final addition, tubes were mixed by gentle tapping, centrifuged, and incubated at 41° C during 90 minutes. Reactions were stopped by storing them at -20° C.

Example 4: Analysis of NASBA amplified reaction products by enzyme-linked gel assay (ELGA).

For enzyme-linked gel assay (ELGA) analysis, NASBA reaction products were hybridised to a target RNA-specific horseradish peroxidase (HRP) 5'-labelled oligonucleotide probe by mixing 2 µl of a NASBA reaction with 1 µl 5x SSC [1x SSC is 150 mM Sodium chloride; 15 mM Sodium citrate], 1 µl concentrated loading buffer [25% (v/v) Glycerol; 10 mM Sodium phosphate buffer (pH 7.0); 0.05% Bromophenol blue; 0.01% Xylene cyanol], and 1 µl HRP-labelled oligonucleotide stock solution (containing about 10^{10} molecules per µl), followed by incubation at 41° C during 15

minutes. After hybridisation, half of the reaction mixture was directly applied onto a 7% polyacrylamide gel supplemented with 0.04% (w/v) dextrane sulphate. After separation of bound and unbound HRP-labelled oligonucleotides by electrophoresis, the probe was visualised in the gel by direct staining with 50 ml Substrate solution [125 mg 3,3',5,5'-tetramethylbenzidine per ml; 0.003% Hydrogen peroxidase: 100 mM Sodium citrate buffer (pH 5.2)] for about 10 minutes at room temperature. Finally, the gel was fixed by overnight incubation in 50% (v/v) methanol solution and air dried.

Example 5: Analysis of NASBA amplified reaction products by ECL detection.

For electrochemiluminescence (ECL) based analysis, detection reagents were prepared by vortexing a capture probe solution, containing biotinylated capture probe immobilised on streptavidin-coated paramagnetic beads (Dynal), until an opaque solution was formed and subsequently mixing 10 µl of this suspension (containing $3.4x10^5$ beads loaded with capture probe) and 10 µl of an ECL labelled probe solution (containing $3.4x10^{11}$ molecules of ECL labelled probe) into fresh reaction tubes. To these mixtures, 5 µl of an appropriately diluted NASBA reaction was added and incubated during 30 minutes at 41° C. Typically, 20-fold diluted reaction mixtures were used. During hybridisation, the tubes were mixed every 10 minutes. Subsequently, 300 µl NASBA QR System Assay Buffer (Organon Teknika) was added to each hybridisation tube and the tubes were positioned in a NASBA QR System for automated reading of ECL signals.

Example 6: Length of the upstream primer elongation.

To determine the possible influence of the length of a non-target specific extension of the upstream primer (P2 primer) on NASBA amplification, U1A SC-RNA, an artificial in vitro generated RNA consisting of a 132 nucleotides HIV-1 fragment (comprising nucleotides 1015 to 1146 of the 5' noncoding region of pv22; see Muir et al., J. Clin. Microbiol. 31., pp31-38 (1993)) flanked by parts of the mRNA encoding the U1 small nuclear ribonucleoprotein (snRNP) particle-specific A protein (U1A), was used in a model system. A P2 primer encompassing 23 nucleotides of the U1A mRNA sequence (nucleotides 378 to 400; see Sillekens et al., EMBO J. 6, pp 3841-3848 (1987)) was synthesised together with three variants of this primer containing

elongations of different sizes at their 5' ends which were not related to the target RNA (Table 1).

Using each of these four variants of the U1A P2 primer in combination with a U1A P1 primer for NASBA, a dilution series of in vitro generated U1A SC-RNA containing 10^4, 10^3, 10^2, and 10 copies per reaction, was amplified. Reaction products were analysed by enzyme-linked gel electrophoresis (ELGA) employing a U1A-specific HRP-labelled oligonucleotide probe. Results are depicted in Figure 1.
Compared to a normal P2 primer only consisting of a target RNA-derived sequence (P2), no significant influence was observed on the efficiency of amplification when the P2 primer was elongated at its 5' end with a non-target RNA specific sequence, irrespective of the length of the elongation (15, 20 or 24 nucleotides for P2+15, P2+20, and P2+24, respectively). More or less arbitrarily, the extension of 20 nucleotides was chosen for further analyses.

Example 7: Composition of the upstream primer elongation.
Two extensions with an identical length of 20 nucleotides (see Example 1), but with a different primary structure were tested for the amplification of several target RNAs and subsequent detection of the generated RNA amplicons. The primer extensions that were used for the NASBA P2 primers are shown in Table 2.
To compare extensions 20A and 20B (Table 2), standard NASBA primer pairs consisting of a regular P1 primer (with a T7 RNA polymerase promoter sequence) and P2 primer were compared to NASBA primer pairs consisting of the same P1 primer but for which the regular P2 primer sequence at its 5' end was elongated with either extension 20A or 20B.

Target RNA sequences for which this primer pair comparison was made, were the U1A SC-RNA as described in Example 1, the mRNAs encoding the major immediate early antigen (IE1 mRNA) or a phosphorylated structural protein of 67 kDa (pp67) of the human cytomegalovirus (HCMV). Serial 10 fold dilutions of in vitro generated RNA for each of the targets was prepared and, subsequently, aliquots of each dilution were used in amplification reactions employing either the standard NASBA primer pair or the primer pairs with the elongated P2 primers (P2+20A, P2+20B).

Upon amplification, the RNA amplicons were analysed by electrochemiluminescence-based detection, using a capture probe and an ECL-labelled detection probe which both were complementary to the target RNA-derived amplicon sequences. As such, for each target RNA the amplicons generated by the different primer pairs could be analysed in the same detection format. Results for the different target RNAs are summarised in Tables 3-5.

From the scores in Table 3 it can be deduced that for U1A SC-RNA the primer pairs with the elongated P2 primers (P1/P2+20A and P1/P2+20B) perform similar to the standard primer pair (P1/P2) in a NASBA-based amplification. In this particular example, target RNA input levels of 10^3 molecules or more revealed a positive test result in all cases, irrespective of the primer pair that was used, whereas 100 molecules of target RNA could be amplified by either primer pair in one out of two cases.

In Tables 4 and 5, comparison of different primer pairs for the amplification of two CMV mRNAs is shown.

As for the U1A SC-RNA, also for the two viral mRNAs of HCMV (IE1 and pp67), no negative effect was observed in the amplification efficiency of the P2 primers that could be attributed to the elongation of the primer with either extension 20A or 20B (Table 1).

Next to comparison of primer pairs on in vitro generated target RNAs that were added directly to NASBA amplification reactions, primer pairs were compared for their amplification efficiency on target RNA that was extracted from a background of plasma or anticoagulated whole blood. These kind of matrices are known to contain substances that can be inhibitory to NASBA amplification and coisolation of these inhibitors or at least part of them cannot be excluded. As a model system, in vitro generated potato leafroll virus (PLRV) RNA was used, since this plant virus RNA was anticipated not to be present in plasma or whole blood from healthy human individuals (nor RNAs possibly cross-reacting with the PLRV RNA-derived primers). PLRV RNA in quantities of 10^5, 10^4, or 10^3 molecules was spiked to a mixture of 100 µl plasma in 900 µl Lysis buffer or 1 ml of whole blood in 9 ml of Lysis buffer. Subsequently, total nucleic acid was isolated from the samples using silica-based

extraction (see above). The resulting nucleic acid extracts were amplified in NASBA-based amplification reactions employing either the standard PLRV primer pair or the primer pairs with the elongated P2 primers (P2+20A and P2+20B; see also Table 1). For the analysis of the amplification reaction mixtures by ECL detection, a target RNA-specific capture probe and a target RNA-specific ECL probe were used. For each primer pair, final scores for the different target RNA input levels, which were each tested in duplicate, are summarised in Table 6 (plasma background) and Table 7 (whole blood background).

For the plasma background, no significant differences were observed for the three primer pairs that were tested. Remarkably, upon extraction from whole blood, the P2 primer with the 20A extension (Table 1) in this example appeared somewhat more sensitive than the 20B-elongated P2 primer.

Overall, it can be concluded that in principle the addition of an extra non-target RNA-related stretch of 20 nucleotides to the 5' end of a NASBA P2 primer has hardly if any influence on the performance of this primer in NASBA amplification reactions, irrespective of the primary structure of this primer elongation.

Example 8: P2 primer elongation for detection

The final outcome of the analysis of a given specimen for the presence of a certain target RNA by NASBA-based amplification is the total of amplification and subsequent detection of the amplicons generated from the target RNA. Influence of the P2 primer elongation in the amplification reaction appeared to be negligible (see above). Next, influence on the detection part of the analysis was investigated. Therefore, standard ECL detection employing a target RNA-derived capture probe as well as a target RNA-derived ECL detection probe was compared to ECL detection in which the same target RNA-derived capture probe was used but the target RNA-specific ECL detection probe was replaced by an ECL detection probe that was identical to the primary structure of the P2 elongation.

Amplicons generated for the different target RNAs in the previous example (Example 2, Tables 3-6) using the elongated P2 primers (P2+20A, P2+20B) were reanalysed with the more generic ECL detection format as described above (target RNA-derived

capture probe/P2 elongation-specific ECL probe). ECL signals thus obtained were compared to the ECL signals as generated by the standard ECL detection format (target RNA-derived capture probe/target RNA-derived ECL probe) in the previous example. Results are shown in Tables 8-10.

The results in Tables 8-10 show that in principle specific ECL detection of NASBA amplicons is possible when using a combination of two oligonucleotides, one of which is specific for the target RNA, whereas the other is complementary to a non-target RNA sequence which is introduced into the RNA amplicons by elongation of the P2 primer. Furthermore, Tables 8 and 9 show that this detection can be equal to a standard ECL detection format employing two target RNA-specific oligonucleotides and can be varied with respect to the primary structure of the stretch of nucleotides used for the elongation of the P2 primer.

Results shown in Table 10 demonstrate that occasionally further optimisation of the detection procedure might improve for certain capture probe/ECL detection probe combinations. Hybridisation at a higher temperature and/or the use of another capture probe might solve the problem of an unusually high background as observed for the ECL probe hybridising to the 20B extension when used in combination with the biotinylated capture probe for PLRV RNA-derived amplicons chosen for this experiment (Table 10).

TABLES

Table 1: P2 primers for NASBA amplification of U1A SC-RNA.

Primer	Length	Sequence *
U1A P2	23 nt	5'-CAGTATGCCAAGACCGACTCAGA-3'
U1A P2+15	38 nt	5'-gatgcaaggtcgcatCAGTATGCCAAGACCGACTCAGA-3'
U1A P2+20	43 nt	5'-gatgcaaggtcgcatatgagCAGTATGCCAAGACCGACTCAGA-3'
U1A P2+24	47 nt	5'-gatgcaaggtcgcatatgagtaagCAGTATGCCAAGACCGACTCAGA-3'

*: target RNA-derived segment in capitals; non-target RNA-derived extensions in small characters

Table 2: NASBA P2 primer extensions.

Extension	Length	Composition
20A	20 nt	5'-GATGCAAGGTCGCATATGAG-3'
20B	20 nt	5'-CTGCAGAGGTGAGGTTACGG-3'

nt: nucleotides

Table 3: Comparison of standard P2 primer with elongated P2 primers for U1A SC-RNA.

RNA input (mol./reaction)	Primer pair					
	P1/P2		P1/P2+20A		P1/P2+20B	
	ECL signal	Score	ECL signal	Score	ECL signal	Score
10^4	out of range	+	1.687.647	+	2.024.305	+
10^3	out of range	+	714.354	+	1.537.823	+
10^3	out of range	+	1.606.363	+	1.240.880	+
10^2	1.044.339	+	143.340	+	131	-
10^2	150	-	142	-	1.217.467	+
0	173	-	154	-	177	-

out of range: more than $3x10^6$ ECL counts

Table 4: Comparison of standard P2 primer with elongated P2 primers for IE1 mRNA.

RNA input (mol./reaction)	Primer pair					
	P1/P2		P1/P2+20A		P1/P2+20B	
	ECL signal	Score	ECL signal	Score	ECL signal	Score
10^4	1.606.288	+	1.660.148	+	1.422.528	+
10^3	1.618.527	+	1.612.729	+	1.892.690	+
10^3	1.536.372	+	1.677.485	+	1.802.925	+
10^2	1.626.115	+	1.849.002	+	out of range	+
10^2	13.902	+	1.605.632	+	1.836.328	+
0	385	-	495	-	421	-

Table 5: Comparison of standard P2 primer with elongated P2 primers for pp67 mRNA.

RNA input (mol./reaction)	Primer pair					
	P1/P2		P1/P2+20A		P1/P2+20B	
	ECL signal	Score	ECL signal	Score	ECL signal	Score
10^4	704.871	+	655.922	+	734.416	+
10^3	789.416	+	730.189	+	1.428.602	+
10^3	831.227	+	682.787	+	758.876	+
10^2	778.911	+	1.026.969	+	1.404.119	+
10^2	775.672	+	926.720	+	671.783	+
0	188	-	-64	-	-82	-

Table 6: Comparison of PLRV primer pairs on PLRV RNA extracted from plasma.

RNA input (mol./extraction)	Background (100 ul)	Primer pair					
		P1/P2		P1/P2+20A		P1/P2+20B	
		ECL signal	Score	ECL signal	Score	ECL signal	Score
10^5	plasma	288.224	+	275.708	+	385.263	+
10^5	plasma	1.043.623	+	1.246.091	+	1.082	+
10^4	plasma	496	-	1.515.348	+	437.808	+
10^4	plasma	1.180	+	880.987	+	419.713	+
10^3	plasma	392	-	2.017	+	470	-
10^3	plasma	328	-	334	-	95.309	+
0	plasma	372	-	396	-	366	-

Table 7: Comparison of PLRV primer pairs on PLRV RNA extracted from whole blood.

RNA input (mol./extraction)	Background (1000 ul)	Primer pair					
		P1/P2		P1/P2+20A		P1/P2+20B	
		ECL signal	Score	ECL signal	Score	ECL signal	Score
10^5	whole blood	6.898	+	599.599	+	2.638	+
10^5	whole blood	2.275	+	583.813	+	552	-
10^4	whole blood	355	-	268	-	483	-
10^4	whole blood	741	-	17.557	+	400	-
10^3	whole blood	4.042	+	313	-	312	-
10^3	whole blood	365	-	413	-	311	-
0	whole blood	403	-	394	-	337	-

Table 8: Comparison of ECL detection formats for U1A SC-RNA-derived NASBA amplicons.

RNA input in amplification (mol./reaction)	Primer pair			
	P1/P2+20A		P1/P2+20B	
	Standard ECL	Generic ECL	Standard ECL	Generic ECL
10^4	1.687.747	1.178.174	2.024.305	1.269.658
10^3	714.354	579.933	1.537.823	755.909
10^3	1.606.363	1.186.682	1.240.880	599.066
10^2	143.340	171.653	131	238
10^2	142	206	1.217.467	580.176
0	154	452	177	N.D.

Table 9: Comparison of ECL detection formats for HCMV pp67 mRNA-derived NASBA amplicons.

RNA input in amplification (mol./reaction)	Primer pair			
	P1/P2+20A		P1/P2+20B	
	Standard ECL	Generic ECL	Standard ECL	Generic ECL
10^4	655.922	516.120	734.416	273.221
10^3	730.189	611.626	1.428.602	171.794
10^3	682.787	682.198	758.876	230.946
10^2	1.026.969	467.032	1.404.119	150.446
10^2	926.720	767.522	671.783	153.164
0	-64	-24	-82	-13

Table 10: Comparison of ECL detection formats for PLRV RNA-derived NASBA amplicons.

RNA input in amplification (mol./reaction)	Primer pair			
	P1/P2+20A		P1/P2+20B	
	Standard ECL	Generic ECL	Standard ECL	Generic ECL
10^5	1.619.881	2.342.866	1.431.443	1.527.467
10^4	1.610.150	2.107.491	1.337.609	1.425.625
10^3	988.817	1.896.155	134.311	199.593
10^2	138.628	1.936.461	215	6.498
10^2	95.738	489.490	239	6.658
0	223	237	292	8.007

CLAIMS

1 Method for tagging the reaction products of a transcription-based amplification assay, comprising the steps of adding to a reaction mixture;

a. at least one oligonucleotide that can function as a nucleic acid primer for a transcription-based amplification reaction
b. appropriate enzymes
c. target sequence

and subsequently incubating the mixture at an appropriate temperature for an appropriate amount of time in order to accomplish amplification of at least part of the target sequence characterised in that said at least one oligonucleotide contains a transcribable non-target related sequence element.

2 Method according to claim 1 characterised in that said transcription-based amplification assay is a NASBA assay.

3 Method according to claims 1 and 2 characterised in that two oligonucleotides contain a transcribable non-target related sequence element.

4 Method according to any of the previous claims characterised in that said transcribable non-target related sequence element comprises a nucleic acid sequence selected from the group consisting of SEQ ID # 1-4.

5 Use of an oligonucleotide comprising a transcribable non-target related sequence element in a method according to any of the preceding claims.

6 Kit for detecting nucleic acid amplified in a transcription-based amplification assay using a method according to any of the claims 1-4 comprising a nucleic acid probe hybridisable to the transcribable non-target related sequence element or its complement.

7 Kit according to claim 6 wherein the nucleic acid probe is attached to a detectable label.

8 Kit according to claim 7 wherein the detectable label is an ECL label.

9. Method for counteracting carry-over contamination in transcription based amplification reaction mixtures, said method comprising:
tagging the reaction products of transcription based amplification assay with the method of claim 1, checking further reaction mixtures prior to amplification for the presence of tagged reaction products and, optionally, removing said tagged reaction products from said reaction mixture.

10. Method according to claim 9, wherein said tagged reaction products are removed by reacting them with capture-oligonucleotides comprising a complementary sequence.

11. Method according to claim 10, wherein said capture-oligonucleotides are bound to a solid phase.

12. Method according to claim 11 wherein the solid phase is contacted with the reaction mixture under conditions suitable for binding the tagged reaction products to the capture-oligonucleotides.

13. Method according to claim 11 or 12, wherein said solid phase is particulate.

14. Method according to claim 13, wherein the solid phase is separated from the reaction mixture by filtration.

15. Method according to claim 13, wherein said solid phase comprises magnetic or (super)paramagnetic beads and a magnet is used to separate the solid phase from the reaction mixture.

16. Method for the amplification of nucleic acid using a transcription based amplification reaction wherein the reaction products of said transcription based amplification reaction are tagged according to the method of claim 1.

17. Method according to claim 16, wherein one or more internal standard RNA(s) are co-amplified with the target and wherein said internal standards RNA's each comprise a unique feature to distinguish them from the target (and each other), and comprise the same primer binding sites as the target.

18. Method for the generation of RNA transcripts that can be used as internal standard in a transcription based amplification reaction, whereby an internal standard sequence is tagged with the method of claim 1 and the transcribable non-target related sequence elements are the primer binding sites for the primers used in the amplification reaction in which the internal standards are to be co-amplfied with a target sequence.

19. Method according to claim 18 wherein internal standards are prepared that have a primer A region and a primer B region, said regions also being present in the target RNA, by subjecting generic input RNA to a first transcription based amplification reaction wherein a first and second primer are used and

- said first primer comprises a sequence homologous to the 5' region of the generic input RNA and further comprising a 5' tail, said 5' tail comprising a primer region A homologous to the target RNA,
- a second primer comprising a sequence complementary to the 3' end of the generic input RNA , said second primer further comprising a sequence of a promoter recognised by an RNA polymerase,, thus creating reaction products having the complement of a primer A region at their 3' end

subsequently subjecting these reaction products of the first transcription based amplification reaction to a second transcription based amplification reaction wherein a third and fourth primer are used and

- said third primer comprises the sequence of the primer A region and the sequence of a promoter for a RNA polymerase,
- said fourth primer comprises a sequence complementary to the 3 ' region of the generic input RNA and a sequence homologous to the primer B region, thus generating the internal standards that have a primer A region and a primer B region, said regions also being present in the target RNA.

FIGURE 1

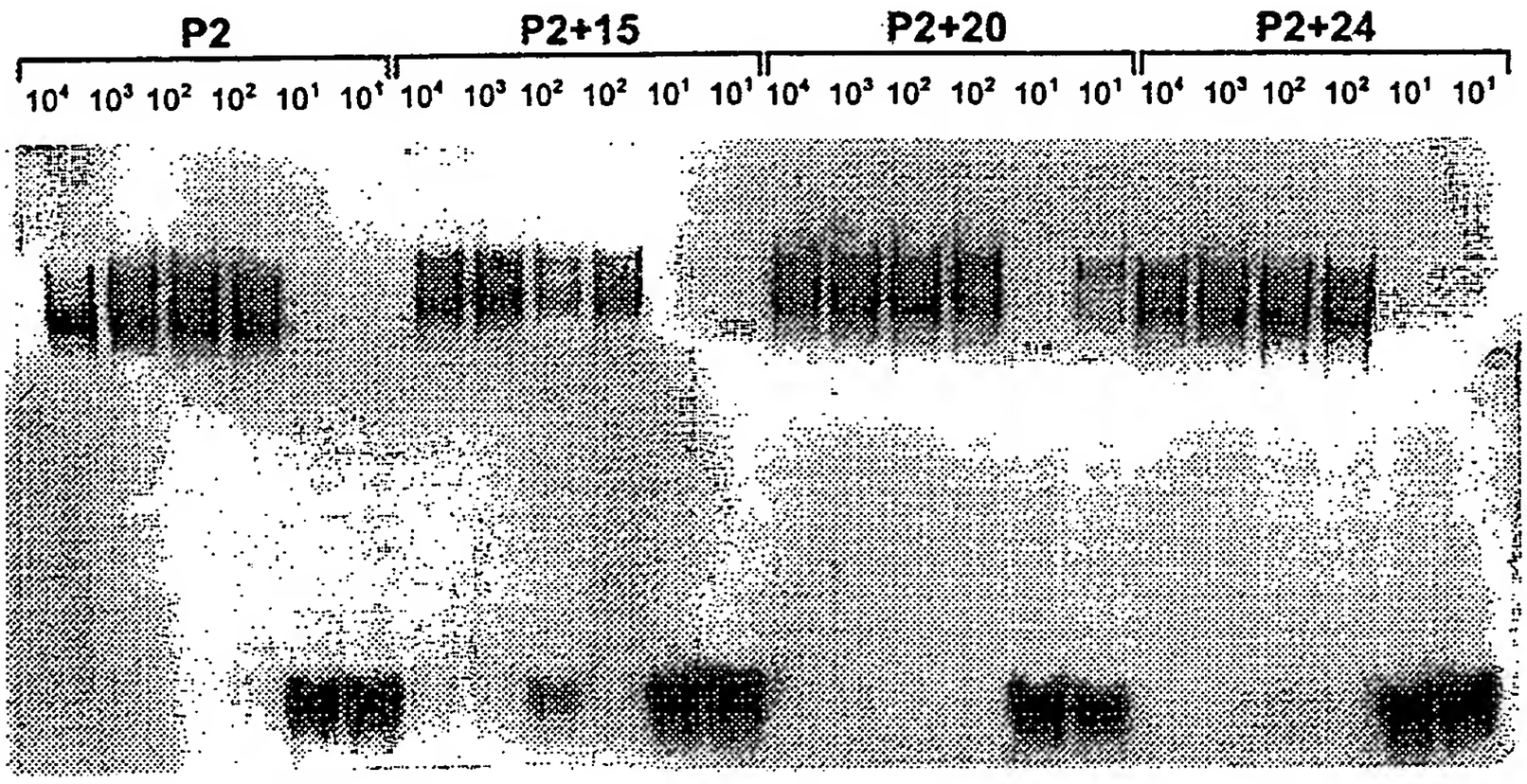
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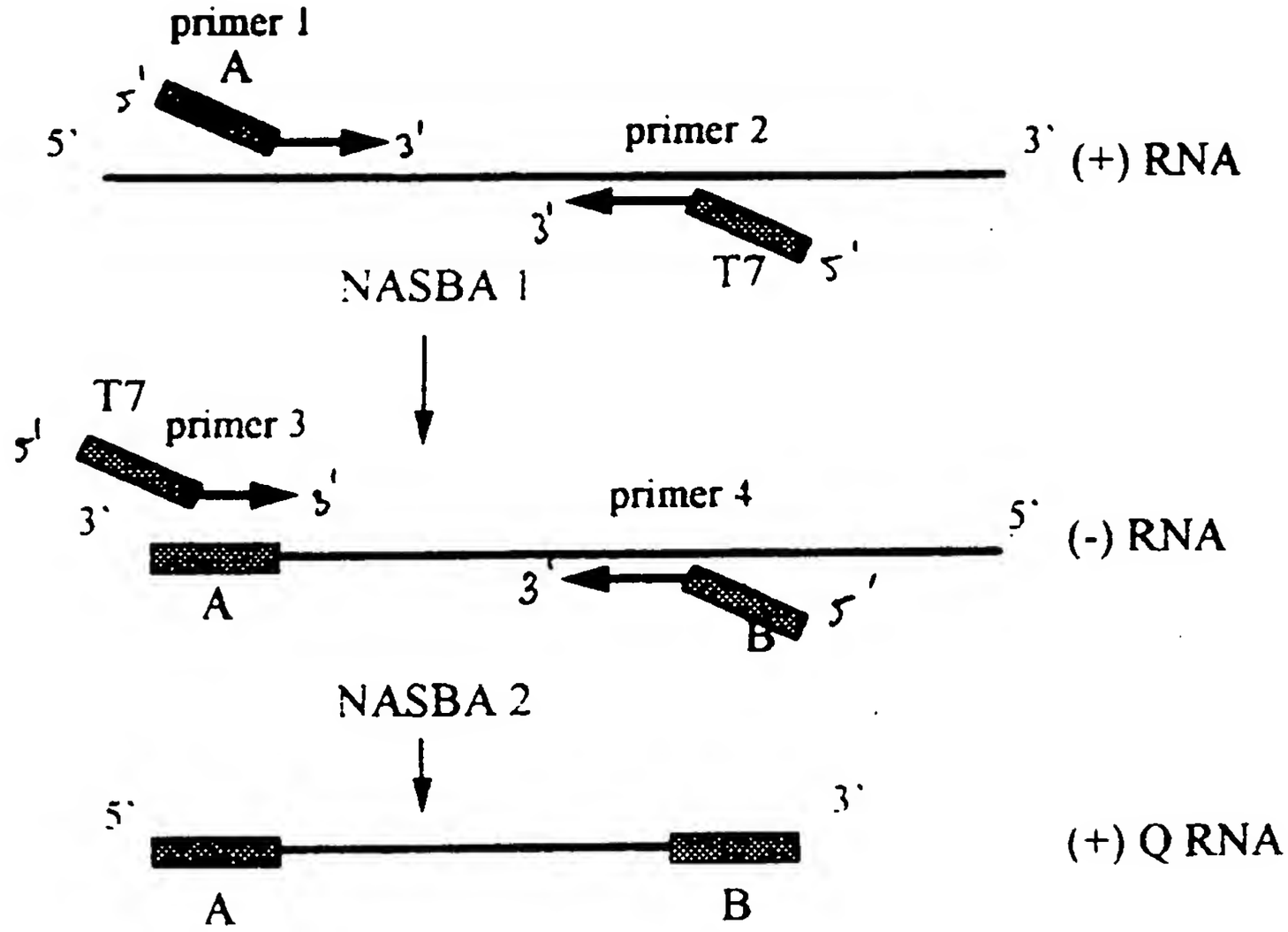
Figure 2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